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Biotechniques for improving acid aluminum tolerance in alfalfa

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Abstract. Alfalfa (Medicago sativa L.), cultivars ARC, Regen Y and Saranac were selected in vitro in a recently developed acid/aluminum toxic media. The new media produced higher initiation rates and higher fresh callus weights than those obtainable with the media described by Meredith and Connor for the selection of aluminum resistant variants in Nicotiana plumbaginofolia. Both rescue and direct initiation yielded adequate amounts of healthy callus for the initiation of embryogenesis. The toxic effect of the acid/aluminum media is expressed in both the percent of explants initiating callus and in the fresh-weights obtained during initiation and two subsequent sub-cultures.

Key words: Alfalfa-Media-Callus-fresh weight

Introduction

Alfalfa (Medicago sativa L.) one of the most important forage legumes in the United States and throughout the world, has been recognized as an aluminum sensitive species. The performance of alfalfa in acidic and infertile soils is very poor (Dudal, 1976). Soil acidity limits plant growth through aluminum (Al) and manganese (Mn) toxicity causing mineral stress (Fageria et al., 1990; Foy, 1988).

Liming can be used to correct soil acidity problems (Rechcigl et al., 1985; Koch and Estes, 1986; Sumner et al., 1986). However, surface application does not neutralize harmful Al in subsurface layers, and mixing lime with subsoils is generally not feasible. Aluminum toxicity is enhanced in soils with low calcium (Ca) saturation and high Al saturation. In addition, soils with similar Ca and Al saturations vary in toxicity according to pH (Wright et al., 1987).

Conventional breeding of alfalfa has made limited progress in inducing Al-tolerance (Devine et al., 1976; Vasil, 1990). In vitro cell selection provides a distinct advantage for adding a single attribute to an otherwise well adapted genotype (Brown and Atannasov, 1984). To allow the expression of Al toxicity in vitro, cell culture media must be formulated by modifying the inorganic composition of standard cell culture formulae. Media must be altered to reduce phosphate and calcium concentration. EDTA must be eliminated. A media proposed for the selection of Al-resistant variants in Nicotiana plumbaginofolia offers these characteristics (Meredith and Connor, 1985). However, alfalfa callus and embryo formation was limited, of poor quality and failed to regenerate in this medium.

A multi-step protocol was chosen to enhance both quantity and quality of callus and embryo formation (Stuart and Strickland, 1984). This process is based on Schenck and Hildebrandt (SH) salts and organics (Schenck and Hildebrandt, 1972). As SH salts are comparatively low in phosphates they can be easily modified for the expression of aluminum toxicity. Therefore a modified SH medium was formulated to study Al-resistant callus formation in alfalfa.

Materials and Methods

The experiment consisted of two media and three treatments. Medium one (SHNaaK) was unmodified SH salts and organics. These included 2500 mg/L KNO_3, 200 mg/L $CaCl_2 \cdot 2H_20$, 400mg/L $MgSO_4 \cdot 7H_2O$, 300 mg/L $NH_4H_2PO_4$, 13200 ug/L $MnSO_4 \cdot 4H_2O$, 1000 ug/L $ZnSO_4 \cdot 7H_2O$, 5000ug/L H_3BO_3, 1000

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ug/L KI, 200 ug/L $CuSO_4 \cdot 5H_2O$, 100 ug/L $Na_2MoO_4 \cdot 2H_2O$, 100 ug/L $CoCl_2 \cdot 6H_2O$, 15000 ug/L $FeSO_4 \cdot 7H_2O$, 20000 ug/L Na_2 EDTA $\cdot 2H_2O$, 100 mg/L myo-inositol, 5000 ug/L thiamine HCl, 5000 ug/L nicotinic acid, 500 ug/L pyridoxine HCl and 3% sucrose. Growth regulators used were 5.0 mg/L naphthaleneacetic acid (Naa) and 2.0 mg/L Kinetin (K). The pH was adjusted to 5.8 with 0.1N NaOH and the medium was filter sterilized. Medium two (SHAlNaaK) consisted of the inorganic and organic components as well as the sucrose and growth regulators found in SHNaaK with the following change: Ca reduced to 11.1 mg/L, EDTA eliminated, reducing the Fe to 1500 ug/L, additional NH_4 ammonium ion was provided by NH_4NO_3 (1.65 gm.), and aluminum supplied at 150 uM/L. The medium was allowed to equilibrate at room temperature for 30 minutes, then the pH was adjusted to 4.65 +/- 0.05 with 0.1N NaOH, followed by filter sterilization.

To insure that the aluminum stayed in suspension, the components were added in the following order and method. To 500 ml millipore water, the macro-elements, micro-elements, vitamins, Fe and NH_4PO_4 were added and each ingredient thoroughly mixed before adding the next. Sucrose, myo-inositol and growth regulators were added and the volume was brought to 900 ml. 150 uM of Al was supplied by 50 ml of a stock solution (1mg/ml) of hydrated $AlSO_4$ added slowly, with rapid stirring. After equilibrating for 30 minutes the pH was adjusted with 0.1 NaOH. The volume was brought to 1000 ml and the media was filter sterilized.

Treatment one was the direct intiation of callus in SHAlNaaK. Callus from the SHAlNaaK was then subcultured twice on SHNaaK. Treatment two, rescue selection, consisted of callus initiated on SHNaaK media, subcultured to SHAlNaaK media, followed by a rescue subculture to SHNaaK. Treatment three consisted of initiation and two subcultures on SHNaaK. All cycles were of 21 days length. Cultures were grown at 27° C with a 16/8 hour light/dark cycle.

Three cultivars were used. Regen Y (RY) was chosen for it's acid/Al sensitivity. Cultivars ARC and Saranac (Sar) were chosen for their relative tolerance shown in field tests.

Explant materials were prepared as follows: Seeds were washed in water containing 2 drops of liquid detergent per 100 ml then rinsed 3 times. Seeds were then washed in 90% ethanol (2 min), soaked for 15 min in 0.85% sodium hypochlorite and rinsed 4 times with sterile tissue culture grade water. Seeds were then aseptically germinated on membrane rafts in magenta boxes (Sigma). After 14 days, magenta boxes with membrane rafts were autoclaved and 50 ml of filter sterilized media was aseptically dispensed into each box. Approximately 1 cm lengths of hypocotyl were dissected and placed into the prepared boxes.

Results

The toxic effect of the SHAlNaaK medium is immediately apparent in the percent of the hypocotyl sections undergoing callus initiation. In unmodified medium ARC,RY and Sar explants initiated callus at rates ranging from 73% to 84% (Table 1). Callus fresh-weights were obtained at the end of three weeks on initiation medium and at the end of each of two three-week subcultures. Data from all treatments was analysed by SAS-ANOVA. Control medium (SHNaak) with reduced iron and calcium did not yield callus weights significantly different than the unmodified SHNaaK.

Table 1.Percent callus initiation on selected media

Cultivar	SHNaaK	SHAlNaaK
ARC	79	50
RY	73	20
Saranac	84	62

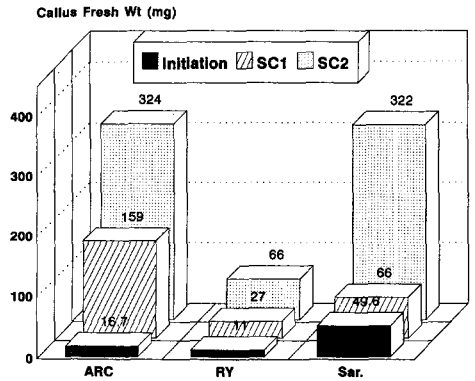

Fig.1. Direct initiation in SHAlNaak. In subculture 1, ARC displayed a significantly greater gain ($p > 0.001$) in fresh weight over Saranac (SAR). At the end of subculture 2 there was no significant difference between ARC and SAR. RY initiated poorly in treatment one and never recovered.

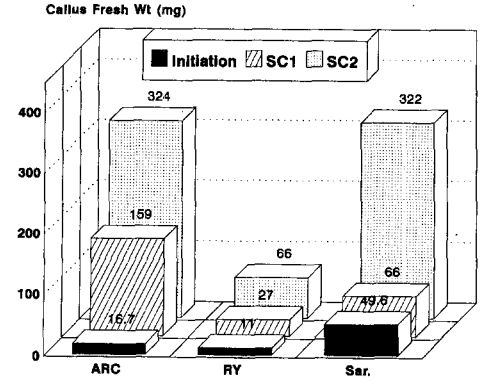

Fig.2. Rescue selection. Treatment 2 yields higher callus weights with all cultivars significantly ($p > 0.001$) different from each other and RY and SAR significantly different from treatment 1 at all three stages. ARC, treatment 2, was significantly different ($p > 0.001$) from ARC treatment 1 at initiation and subculture one, however at the end of the second cubculture there was no significant difference.

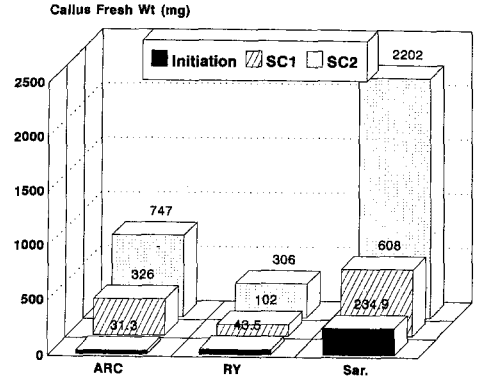

Fig.3. SHNaak initiation and subculture. When treatment three was compared with treatments 1 and 2 at both subcultures, callus fresh-weights were significantly higher ($p > 0.001$) for all cultivars at all subcultures. This reflects the optimum conditions of treatment 3.

Discussion

Comparisons of each cultivar's performance in direct initiation, rescue and unmodified media show that the

media is exerting an aluminum toxic effect. Both direct and rescue selection pathways will produce an adequate amount of callus to proceed with embryo induction and formation. The greater amount of callus is available through rescue selection. However, whether plants resulting from these pathways will display equal tolerance remains to be seen. Cultivar Saranac has shown the most tolerance in vitro. Saranac has a broader genetic background than either ARC or Regen Y which may explain it's greater tolerance. Although complete data is unavailable for analysis, 79 plants produced with this aluminum modified medium are in the field at the USDA-ARS station in Beckley, W.Va. These plants, grown in acid/aluminum soil along witho unselected controls exhibit no readily discernable signs of Al toxicity, ie., yellowing and overall lack of growth. However, control plants display yellowing of the leaves and markedly less vegetative growth. Root and shoot dry-weights are to be assayed soon.

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